REMARKS/ARGUMENTS

Claims 28-29 and 31-54 are pending.

Claims 1-27 and 30 have been cancelled.

Claims 45 and 47-54 have been withdrawn.

Claims 18, 33-36, 38, and 40 have been amended to improve readability.

Support for the amendments is found in the claims and specification, as originally filed. No new matter is believed to have been added.

Applicants wish thank the Examiner for indicating that claims 43-44 would be allowable is rewritten in an independent form.

Claims 28, 31-42, and 46 are rejected under 35 U.S.C. 103(a) over Oh et al., US 5,851,778, Ballerstadt et al., Anal. Cimica Acta, 345:203-212 (1997), and Groopman et al., PNAS, 81:7728-31 (1984). The rejection is traversed because the combination of the references does not describe:

1) The claimed trifunctional reagent;

2) a regeneration of a solid support and carrying 3) and 4) continuously;

3) measuring the intensity of a signal emitted by a luminescent group L on a solid support, which is *proportional* to the amount of an analyte to be detected; and

4) one would not have been motivated to combine the methods of Oh et al., Ballerstadt et al., and Groopman et al. because the references describe different technologies the combination of which is improper.

Subject matter and advantages of the present invention

The invention defined by Claim 28 relates to a method for detection of an analyte a in a fluid sample, the method comprising:

1) saturating a solid support comprising, on at least part of its surface, at least one trifunctional reagent (tripod Y) comprising the following three functional poles:

i) a luminescent group (L),

ii) a molecule (B) chosen from the analyte a, an analog of the analyte a or a fragment of the analyte a; and

iii) a function that provides attachment of the trifunctional reagent to the surface of the solid support, with a receptor for the analyte a, the receptor being labeled with a compound (Q) (receptor-Q) that quenches the luminescence of the group L, thereby forming a complex C between the molecule (B) and the receptor-Q;

2) bringing the solid support obtained in step 1) into contact with a fluid sample that may comprise the analyte a to be detected;

3) measuring the intensity of the signal emitted by the group L, which is proportional to the amount of analyte a present in the fluid sample; and

4) regenerating the solid support by bringing the solid support into contact with the receptor-Q,

wherein 3) and 4) are carried out continuously.

The claimed method is a method for continuous, **heterogeneous-phase detection** of analytes in a fluid sample that makes possible to avoid any incubation, prior to the detection step per se, of the analyte with a labeled antibody or a labeled analyte. In Claim 28, 3) and 4) are carried out continuously.

According to the claimed method:

- step 1) allows complexation of the molecule (B) with the receptor-Q. At the end of this first step, the luminescence of L is decreased or suppressed.

- In step 2), bringing the sample into contact with the surface of the solid support will induce, when said sample contains the analyte a, competition between the analyte a and the molecule (B) for the formation of the complex with the receptor-Q. The binding of the analyte a to the receptor-Q will result in the receptor-Q being eliminated from the surface of

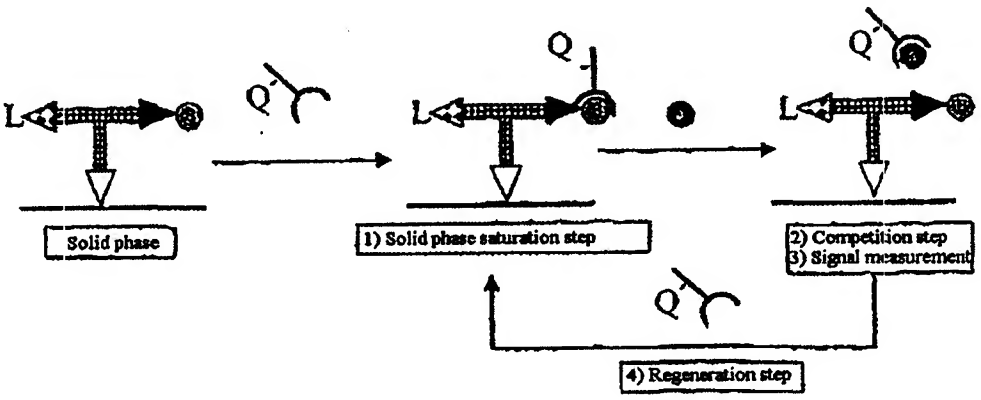
the solid support and in the luminescence emitted by the compound (L) present on the tripod Y being restored.

- The intensity of the signal measured in 3) is then proportional to the amount of analyte a present in the sample to be analyzed.

- The regeneration of 4) will again bring about complexation of the receptor-Q on the tripod Y and thus suppression of the luminescence of L, thereby allowing further detection of the analyte a in a new sample. **No intermediate washing steps are necessary between 3) and 4).**

The principle of the method for the detection of the analyte a in accordance with the claimed method is represented diagrammatically in scheme A below:

SCHEME A



The method of detection in accordance with the invention has a large number of advantages:

1) due in particular to the specific structural conformation of the tripod Y used during the method, the step consisting of the **regeneration of the solid phase can be carried out very readily**, without any alteration of its properties. In fact, in all the flow assays described according to the claimed method, the signal is measured after the formation of a complex bound to the solid phase. Consequently, and even though some of the assaying methods

described by the prior state of the art allow several successive assays, they are however limited in number and require a solid support regeneration step that is often long and restricting, which results in the dissociation of the complex formed. In addition, the drastic conditions for carrying out these regeneration steps (passing over acid or basic solutions) mean that the detection method must be stopped, thus prohibiting any application of these methods to **continuous assays of a given analyte**.

In the claimed method, the presence of the analyte a in the sample brings about dissociation of the receptor-Q and of the molecule (B); **the regeneration step therefore consists quite simply in reforming this complex by adding receptor-Q**. This regeneration step does not therefore involve the use of acid or basic solutions that may impair the properties of the molecules of the solid phase or an exchange reaction between two molecules at the receptor binding site, the kinetics of which are longer than the reaction to form a complex,

2) the signal is measured in the region on which the tripod Y was immobilized, which makes it possible to obtain a localized signal, unlike the assays developed according to the prior art, in which the signal, bound to molecules in solution, is measured at the outlet of a capillar,.

3) since the signal is localized, several molecules may be detected simultaneously on the same solid support by attaching, to distinct and known zones thereof, several types of tripods Y that differ from one another through the nature of the molecule (B) that they comprise,

4) the signal measured corresponds to all the molecules of analyte a that have been in contact with the solid support between two regenerations. This particularity of the method in accordance with the invention allows permanent monitoring to be obtained while at the same time taking measurements that are spaced out over time,

5) this assay format is applicable to all molecules since it does not require the simultaneous binding of two receptors to the analyte, as is often necessary according to the assay methods previously known, and which require the analyte to be of a sufficient size.

6) the presence of analyte a in the sample results in the appearance of a signal, unlike most of the competition assays known from the prior art, the appearance of a signal allowing easier detection,

7) finally, since the detection system uses the energy transfer phenomenon, it also makes it possible to detect and to quantify the presence of an analyte a by means of the variation in luminescence of the compound Q if it is fluorescent or the variation in the apparent time for decrease in luminescence of the compound (L).

Disclosure of the cited references

(a) Oh et al. teach a trifunctional conjugate comprising three chemical moieties attached through a spacer moiety. The method of use of those conjugates relate on the principle of steric hindrance.

In the Oh et al. assay, a sample is contacted with the conjugate, a limited quantity of analyte binding partner and an excess of small molecule binding partner. A presence of the analyte is determined by detecting the amount of an analyte binding partner diverted away from the analyte attached to the spacer of the conjugate (abstract).

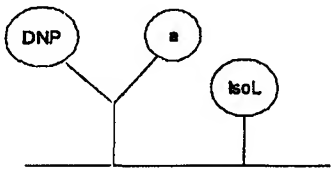
Most of the embodiments of Oh et al. are concerned with conjugates in solution which are not relevant as prior art to the claimed invention.

A variant including a solid support is disclosed in figure 11, col. 18, 1, 20-50 and example 8.

Two of the tridentate members are small molecules ligands such as theophylline and DNP. The third tridentate member is a solid support. According to that variant, it should be

noted that the luminescent group (L) is directly linked to the solid support and not to the trifunctional reagent.

The variant of figure 11 works according to the following scheme:



Scheme A

The solid support is functionalized by a tridentate conjugate comprising an analyte (a) (theophylline), a small molecule ligand DNP and a function that links the trifunctional reagent to the solid support.

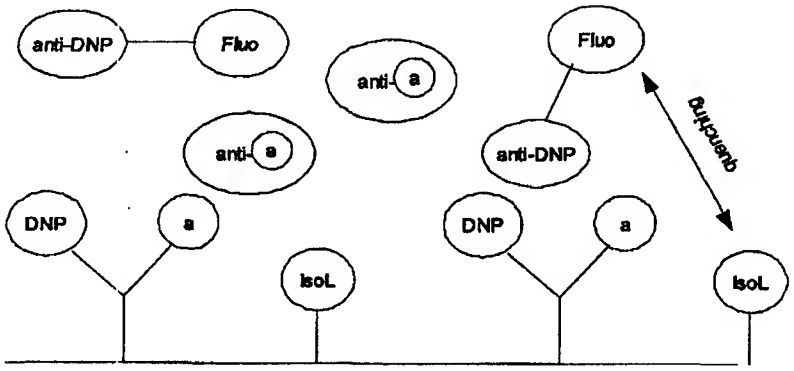
A luminescent molecule (IsoL) or isoluminol is directly linked to the solid support (col. 18, 1, 39-45).

Thus, it cannot be said that the reagent comprises a luminescent group.

A proximity label, like a luminescent molecule fluorescein, is attached to the DNP antibody.

The fluorescein-DNP antibody and a theophylline antibody are incubated with the functionalized solid support.

Scheme B



The anti-a antibody and the anti-DNP antibody are in competition to attach to their respective ligands. At equilibrium, a certain quantity of each antibody is attached to its respective ligand.

Where the anti-DNP-Fluorescein ligand is attached to DNP, the fluorescein quenches the isoluminol luminescent molecule. Steric hindrance prevents the attachment of both antibodies to one sole trifunctional conjugate.

Then the analyte a to be detected is introduced.

There is a competition between the analyte a in solution and the analyte of the tridentate.

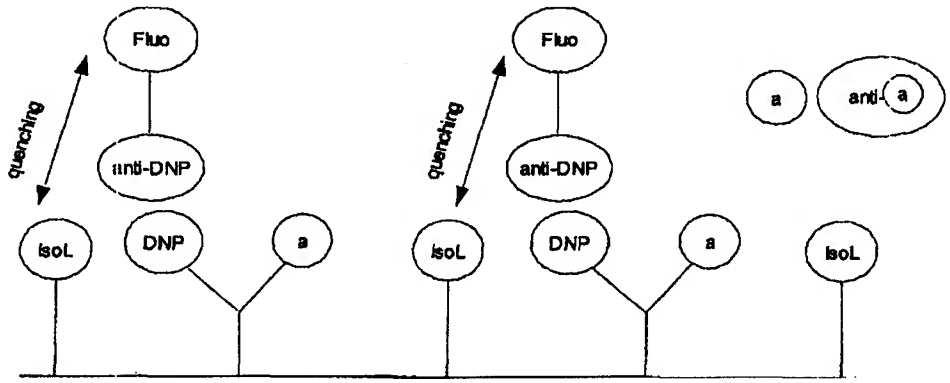
A new equilibrium must be reached:

Anti-a antibodies liberate a ligands comprised in the tridentate, and link to the a in solution. The liberated tridentates are free to conjugate to anti-DNP antibodies.

More anti-DNP antibodies are linked to their DNP ligands. As DNP antibodies bear a luminescent molecule, this results in more quenching of the isoluminol. So that the

luminescence is inversely proportional to the concentration of the analyte (a) (theophyllin).

Scheme C



It can be noted that numerous steps are necessary to perform such a measurement: optimum reagent concentrations have to be determined (anti-a antibody and anti-DNP antibody - see col. 38, 1, 22 - col. 39, 1, 5).

It is specially indicated that in the case wherein a solid support is used incubation times have to be extended (col. 39, 1, 35-52).

Finally, no indication is given regarding a possibility of regenerating the solid support.

When the method of Oh et al. is compared to claim 1, the following comment can be made:

Oh et al. relies on the steric hindrance to reach equilibrium between conjugates. The result, as already detailed above, is that the signal is inversely proportional to the quantity of analyte.

This is a significant difference with the claimed method wherein the intensity of the signal emitted by L is proportional to the amount of analyte.

Moreover, bringing the solid support into contact with the (•) -antibody would not regenerate the solid support:

For each experiment an optimum reagent concentration for energy transfer must be reached (see col. 38, 1n. 22 and following). Adding anti (•) antibody would not permit restoration of that equilibrium.

It should be stressed that the necessity of determining these equilibrium conditions makes the method very long and complicated.

Although no indication is given, it is apparent that the regeneration only can be obtained by the complete washing of the solid support.

Thus, a continuity between steps 3) and 4) is not possible with the method of Oh et al.

To summarize, the most important differences between Oh et al. and the invention are listed below:

- The intensity of the signal measured in Oh et al.'s solid support variant is inversely proportional to the presence of the analyte to be detected.

- The step of regeneration of the solid support of Oh et al. cannot be performed continuously with steps 3) by bringing the solid support into contact with anti antibody.

(b) The Examiner has relied on **Ballerstadt et al.** for the missing characteristics.

However, combining Oh et al. and Ballerstadt et al. is improper and would not lead to the claimed method for the following reasons:

Combining Oh et al. and Ballerstadt et al. is improper because Oh et al. is based on a detection system grafted on a solid support, while Ballerstadt et al. describe a homogeneous method of assaying, i.e., a method carried out in a solution.

Additionally, Ballerstadt et al. do not describe a signal proportional to the analyte A.

Ballerstadt et al. do not teach regeneration of the system which they describe.

Ballerstadt et al. even admits that they have not found conditions of the compromise that would have permitted obtaining a significant fluorescence signal without precipitating and distorting the equilibrium of the system.

As Ballerstadt et al. have failed to adapt the described system to a continuous configuration, in a hollow fibber, one skilled in the art would not have been motivated to combine the assays of Oh et al. and Ballerstadt et al.

Ballerstadt et al. disclose a fluorescence quenching affinity assay. The assay is based on the use of an unspecific lectin and two fluorochrome-labeled dextrans bearing specific ligands which bind to the same receptor as the receptor of the analyte to be detected (Abstract).

In the absence of analyte A dextran-lectin-dextran complexes are formed.

Contrarily to the simplified scheme that the abstract discloses, the publication does not conceal that, even in the absence of the (sugar) analyte A, the situation in the solution is more complex than described in the abstract.

Specifically, Figure 1 only represents the P_FRP_R complex, but the text starting p. 204, col. 2, paragraph 2 clearly describes that lectin R acts as a crosslinking receptor and when R has two binding sites, it takes part to the formation of six complexes according to reaction schemes disclosed on p. 205, col. 1, paragraph 1.

Only the P_FRP_R complex (reaction 6) is a quenching effective complex.

Although the Receptor protein R is said to be bivalent in figure 1, only some limited cases of complexes formation are illustrated.

When the lectin is Co A, the lectin has four receptor sites and each site can be left free or complexed by P_F or P_R so that the number of complexes that may form around one lectin is $4 \times 3 = 12$.

The authors do not conceal that "the exact analysis of these multivalent interactions will be very complex" (p. 205, col. 1, paragraph 2).

When, for example, a complex $P_FP_FRP_R$ is formed, one can easily understand that the quenching occurs, but only in a partial manner.

In addition to this complexity, it is stated (p. 205, col. 1, paragraph 3) that in the case of the bivalent R, six dissociation constants are requested to establish the relative amounts of each of the complex species.

This is the situation before introduction of analyte A.

Then in the presence of a specific sugar A, which competes with the fluorochrome labeled dextrans for the lectin binding sites, it is stated that the dextran complexes will be replaced by sugar/lectin complexes (p. 205, col. 1, paragraph 4).

However, it should not be forgotten that the initial mixture comprises more than one species. When one considers the simplest case, with R as a bivalent lectin, one can find in the initial mixture the following species:

R, RP_F, RP_R, P_RRP_R, P_RRP_F, P_FRP_F.

Each of these six species can react with A, so that, in addition to these six species, after introduction of the analyte A, one will find: RA, ARA, ARP_F, ARP_R.

Any of these four additional species can result from the displacement of one or two ligands from any of the six initial species, which makes $6 \times 4 = 24$ possible reactions.

For each of these displacements, a specific equilibrium constant exists.

Only one initial species P_FRP_R is characterized by fluorescence quenching.

Only the following reactions give a change in fluorescence:

$P_FRP_R + A \rightleftharpoons P_FRA$

$P_FRP_R + A \rightleftharpoons P_RRA$

It is true that when A is introduced into the mixture, a decrease of quenching must occur, but it is incorrect that this decrease is proportional to the amount of analyte A in the sample. As has been demonstrated above, so many equilibrium constants have to be taken into account to determine the equilibrium situation before and after the introduction of A, that it is absolutely impossible to speak of proportionality.

The simplest of the system disclosed in Ballerstadt et al. is when at the equilibrium before A is introduced, which relies on six dissociation constants, and at least two additional dissociation constants must be taken into account when A is added.

No simple calculation can be derived from such a complex system, and certainly not proportionality.

This analysis is a simplification of what actually occurs in the mixture, because the authors relevantly speak of crosslinking of dextran by lectin, which means that even more complex species will be formed in the mixture and such species are of the oligomer or polymer type, and have actually been observed: "we estimate that almost 100% of the dextran occurs in the desired crosslinked form (PRP or $PRP)_n$) (p. 211, col. 1, 1. 13-14 starting from the end).

Quenching and de-quenching in $(PRP)_n$ oligomers when A is added results from complex reactions and equilibrium displacements and cannot be quantified.

A precipitations is observed in some cases of crosslinking of dextran by lectin (p. 207 figure 3, p. 203, col. 1) which makes the resulting fluorescence quenching and de-quenching irregular. After the addition of glucose (analyte), no proportionality is observed. Unspecific quenching between both forms of dextrans is also mentioned (p. 208, col. 2, paragraph 1).

Even when the precipitation does not occur, e.g., the experiment illustrated by figure 7, the curve is clearly asymptotic, not linear, showing that the word "proportional" has been used abusive by the authors in the abstract.

Nowhere in Ballerstadt et al. is a proof of proportionality can be found. On the contrary, the authors insist on the complexity of the system, and the difficulty to equilibrate the system.

Even when one of the reactants, R for example, was linked to a solid surface to make a solid support system, there would be no teaching regarding proportionality of a signal to the presence of an analyte that could be derived therefrom.

Another factor that would have dissuaded the skilled professional of using a system taught by Ballerstadt et al. in a solid surface device is the problem of mobility of macromolecules.

Ballerstadt et al. have observed that when the assay is conducted in a hollow libber, in contrast to an assay in a cuvette, the fluorescence recovery is extremely low (2% compared to 30%). It is explained by the low response with the lack of agitation of the system (p. 203, col. 2, paragraph 2). Thus, it can be deduced that such a system can be of interest when in an agitated reactor, that is to say in solution, but would provide absolutely no significant response in a semi-static system, like the hollow fibber configuration test has proved. Such a result, from a comparative experiment, would have dissuaded the skilled professional to adapt the Ballerstadt et al. system to an even more static configuration, like the grafting of one of the reactants, the lectin R or a dextran, to the walls of a solid support.

Thus, one would not have been motivated to adapt the Oh et al. system to the Ballerstadt reactants with a reasonable expectation of achieving the claimed method.

The Examiner has alleged that, as the reaction between the receptor labels and the analytes are termed "elementary reversible reactions", the Ballerstadt et al. system can work as a reversibly working sensor.

However, this assertion is not supported by the specificities of Ballerstadt's system:

As has been explained above, Ballerstadt et al. dissuades the skilled professional to use their system in a continuous process; only the cuvette assays can work, the hollow fibber test gave non significant results (p. 209, col. 2). Thus, the principle on which the claimed method is based, i. e., regenerating the system by simply rinsing with a solution of receptor-Q, is not feasible based on Ballerstadt et al.

Actually the cuvette system is a batch system.

An addition of further quantities of dextran in the system, after a first complete run has been performed in the Ballerstadt et al. system, would have disturbed the equilibrium, but it would have also provoke dextran precipitation, as has been observed by Ballerstadt et al. (p. 207, col. 2 and fig. 3). Thus, the system would have been completely desequilibrated and non usable for a further test.

Ballerstadt et al. do not describe recycling and the problem of precipitation of dextan prevents any addition of this reactant beyond a certain point. The authors describe that it is necessary to maintain the lectin in excess of dextran (p. 21.0, col. 2, last paragraph):

"In order to simultaneously prevent precipitation and obtain a measurable fluorescence signal, the absolute lectin concentration had to be kept low but still at an excess of dextran in molar terms. At low ratios, the lectin/dextran complexes are smaller in size, thus, their segregation from the solution is strongly delayed. Under those conditions, the overall fluorescence quench was fairly low (approximately 5%). At this low fluorescence signal, one can imagine that the sensor response would be prone to non-specific influences when being used in biological solutions."

The conclusion is extremely significant:

"However, the significant challenge is to improve the strength and the stability of the fluorescence signal without compromising the reversibility of the assay." (p. 211, col. 2, last two lines - p. 212)

Reversibility means that the system is at equilibrium, not precipitated.

The authors admit that they could not obtain a significant fluorescence signal without precipitation.

Not only Ballerstadt et al. have not mentioned a regeneration of the system for continuous testing, but they even admit that the compromise between the obtaining a significant fluorescence signal and an equilibrium state has not been found.

Consequently, the skilled professional would not have relied on Ballerstadt et al. for means to improve Oh et al. with regards to regeneration.

(c) The Examiner has relied on **Groopman et al**. for regenerating a support.

However, combining Groopman et al., Oh et al. and Ballerstadt et al. is improper and would not have resulted in the claimed.

Groopman et al. describe a method for the regeneration of monoclonal antibody affinity columns. An antibody is covalently bound to a sepharose column and used in an immunosorbent assay.

The regeneration of the column is detailed p. 7730, col. 2, 1st paragraph: "Sequential washes with phosphate buffer (pH 3.0), diethanolamine buffer (pH 9.9), and phosphate buffer (pH 2.0) removed <1% of the applied [3H]AFB_1. However, quantitative elution of the bound [3H]AFB_1 was achieved by using 50% dimethyl sulfoxied (Me_2SO) in phosphate buffer (pH 7.4) (50% Me_2SO buffer). The antibody column was regenerated by washing the column with P_i/NaCl (pH 7.4)."

Such a treatment in rather severe acidic conditions is what the skilled professional would actually use to regenerate the Oh et al. solid support.

However, such a treatment is different from that claimed (i.e., bringing the solid support into contact with the receptor-Q), and applying this treatment between 3) and 4) contradicts the characteristic "when 3) and 4) are carried out continuously."

As has been explained previously, Oh et al.'s method requires the reaching of an equilibrium, of the reagents (antibodies to the two ligands). It cannot be combined with a thorough washing of the column in a manner which could be qualified as "continuous".

Thus, combining the assays of Oh et al. and Groopman et al. is improper because Oh et al.'s method requires the reaching of an equilibrium of the reagents, while Groopman et al. describe a thorough washing of a column.

A combination of Oh et al. and Groopman et al. certainly does not result in the claimed regeneration.

Thus, the claimed method is not obvious over Oh et al., Ballerstadt et al., and Groopman because the combination is improper as these methods are fundamental different and even when combined certainly do no result in the claimed method.

Applicants request that the rejection be withdrawn.

Claims 29 is rejected under 35 U.S.C. 103(a) over Oh et al., Ballerstadt et al., Groopman et al., and Plowman et al., Anal. Chem., 71:4344-52 (1999). The rejection is traversed because the combination of the references does not describe:

(1) a trifunctional reagent;

(2) a regeneration of a solid support;

(3) measuring the intensity of a signal emitted by a luminescent group L on a solid support, which is proportional to the amount of an analyte to be detected; and

(4) one would not have been motivated to combine the references with a reasonable expectation of success because the references describe different technologies the combination of which is improper.

The disclosures of Oh et al., Ballerstadt et al., and Groopman et al. are described above.

Plowman et al. do not cure the deficiency. Plowman et al. describe a sandwich immunoassay for detecting multiple analytes (fig. 1) in which a capture antibody is attached to a solid support and a pre-mixed analyte with a tracer antibody labeled with a dye is passed over the capture antibody (fig. 1; Material and Methods, pages 4346-47).

Plowman et al. do not describe tripoids as claimed and a regeneration of a solid support ("regeneration of the surface was not considered," page 4347, right col., line 10). Thus, substituting the Plowman et al. assay into that of Lee et al. and Oh et al. assay still does not produce the claimed method.

In addition, Plowman et al. describe an assay based on a completely different principle (e.g., integrated optical waveguide used to evanescently excite fluorescence from a multianalyte sensor surface in a sandwich assay; multiple channels; pre-mixed analytes and labeled tracer antibodies) and uses a different set up and reagents compared to Oh et al. and Lee et al. so that detecting multiple analytes in the Oh et al. and Ballerstadt et al. assay by using the principle of Plowman et al. is not possible.

Thus, Oh et al., Ballerstadt et al., Groopman et al., and Plowman et al. do not make the claimed method obvious.

Applicants request that the rejection be withdrawn.

A Notice of Allowance for all pending claims is requested.

Respectfully submitted,

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